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PCR Identification of *Mycobacterium bovis* BCG

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The attenuated bacillus Calmette-Guérin (BCG) vaccine strain is derived from a virulent strain of *Mycobacterium bovis*. BCG is difficult to differentiate from other strains of *M. bovis* and other members of the *M. tuberculosis* complex by conventional methods. Recently, a genomic region designated RD1 was found to be present in all virulent *M. bovis* and *M. tuberculosis* strains tested but deleted from all BCG strains tested. With this information, a multiplex PCR method was developed to detect the RD1 deletion. A large collection of BCG and other *M. tuberculosis* complex strains from diverse host and geographic origins was tested. RD1 was deleted in 23 of 23 BCG strains. RD1 was present in 129 of 129 other *M. tuberculosis* complex strains. This multiplex PCR method can be used as a tool for the rapid and specific identification of BCG.

Bacillus Calmette-Guérin (BCG) is an attenuated derivative of a virulent strain of *Mycobacterium bovis*. BCG has been used as a vaccine against *M. tuberculosis*, as a recombinant vehicle for multivalent vaccines against other infectious diseases, and as cancer immunotherapy (3, 20). BCG can cause disease in humans, especially those with cellular immunodeficiencies (16, 18, 19). Therefore, the ability to rapidly and specifically identify BCG is clinically important.

BCG is genetically and phenotypically similar to other strains of *M. bovis* and to other species of the *M. tuberculosis* complex (*M. tuberculosis*, *M. africanum*, and *M. microti*). The combination of biochemical and growth features can strongly suggest that an isolate is *M. bovis* BCG, but none is definitive (29, 30). Mahairas and his colleagues employed subtractive genomic hybridization to identify genetic differences between virulent strains of *M. bovis* and *M. tuberculosis* and avirulent BCG (21). One region of difference, designated RD1, was shown to be absent in 6 BCG strains and present in 1 *M. bovis* strain and 62 *M. tuberculosis* complex strains. Because the RD1 region appeared to have potential as a specific marker for BCG, we developed a multiplex PCR that targets the RD1 region. To evaluate this method for the rapid and specific detection of BCG, a large strain collection, including multiple representatives of *M. bovis* BCG, non-BCG *M. bovis*, and other species of the *M. tuberculosis* complex, was analyzed.

MATERIALS AND METHODS

Sources and identification of strains. DNA samples from 152 strains of the *M. tuberculosis* complex were used in this study. The strains are listed in Table 1. The sources, phenotypic characteristics, and methods of species identification are described in the references cited in Table 1.

DNA preparation. Strains were killed by heat or ethanol fixation (31). DNA was released from cells by agitation with glass beads (11) or by snap freeze-thawing (32). DNA samples were coded.

PCR methods. The following PCR primers were used: ET1, 5′-AAGCGGTT GCCGCCGACCGACC-3′; ET2, 5′-CTGGCTATATTCCTGGGCCCGG-3′; and ET3, 5′-GAGGCGATCTGGCGGTTTGGGG-3′. PCR was performed with 5 μl of each coded DNA sample in a total volume of 50 μl of PCR mix. The PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% (wt/vol) gelatin, 2 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer Cetus, Norwalk, Conn.), 5 pmol each of primers ET1 and ET3, and 25 pmol of ET2. The mixture was denatured for 3 min at 95°C and cycled 40 times to 94°C for 30 s and 65°C for 1 min, followed by a final 10-min extension at 72°C. One interspersed negative control (reagents only; no DNA) was included for every three samples tested. The positive controls were 200 pg each of chromosomal DNA from *M. tuberculosis* TMC 107 (Erdman) and *M. bovis* ATCC 35734 (BCG Pasteur). PCR products were separated by electrophoresis on a 3% agarose gel in Tris-boric acid-EDTA buffer. The presence and size of each PCR product were determined by UV transillumination of the ethidium bromide-stained gel by two independent readers who were blinded to strain identity (E.A.T. and R.F.).

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RESULTS

Figure 1 shows the multiplex PCR design, including the three primers and DNA targets. Primers ET1 and ET3 are complementary to regions flanking the RD1 sequence. In strains without RD1, these primers bind and amplify a 200-bp region. In strains with RD1, these primers bind but the 9,650-bp sequence is too large to efficiently amplify. Primer ET2 is complementary to DNA within the RD1 sequence. Therefore, ET2 and ET3 yield a 150-bp product only in strains with part or all of the RD1 sequence present.

PCR conditions were optimized separately with each primer pair. The *M. bovis* type strain DNA consistently yielded a single 150-bp product with primers ET2 and ET3, indicating that at least a portion of RD1 was present. The *M. bovis* type strain DNA yielded no product with ET1 and ET3. *M. bovis* BCG Pasteur consistently yielded a single 200-bp product with primers ET1 and ET3 and yielded no product with primers ET2 and ET3, indicating deletion of the RD1 sequence.

Multiplex PCR amplification products were obtained from 152 of 152 *M. tuberculosis* complex strains. The 150- and 200-bp products were easily distinguished on a 3% agarose gel (Fig. 2). Table 1 lists the size of the product from each strain. Several strains were tested repeatedly, in some cases with different DNA preparations. Results were always reproducible, and there was complete concordance between the two researchers who interpreted the gels.

Twenty reference and three clinical strains of BCG were analyzed by the multiplex PCR method. The reference strains included early substrains (ATCC 35736, TMC 1009, TMC 1019, and TMC 1022) and late substrains. RD1 was deleted from all BCG strains tested. The RD1 sequence was present in 55 of 55 strains of virulent *M. bovis* and 74 of 74 other *M.*

TABLE 1. Strains of *M. tuberculosis* complex analyzed by multiplex PCR

Species	Description (no. of strains)[a]	Geographic location(s)[b]	Size of PCR product (bp)	Reference(s)
M. tuberculosis	TMC 102, type strain, human isolate (1)	New York	150	22
	TMC 201, avirulent derivative of TMC 102 (1)	New York	150	22
	TMC 107, Erdman, human isolate (1)	Minnesota	150	22
	Human isolates (5)	Arkansas	150	4
	Human isolates (4)	Korea	150	10
	Human isolates resistant to rifampin (43)	British Columbia, Colorado, New York, Ontario, Texas, Belgium, Burundi, Honduras, Japan, Peru, Philippines, Rwanda, Switzerland, Vietnam, Yemen	150	32
M. africanum	ATCC 25420, type strain, human isolate (1)	Senegal	150	2
	Human isolates (17)	Sierra Leone	150	32
M. microti	ATCC 19422, type strain, field vole isolate (1)	England	150	2
M. bovis, non-BCG	TMC 410, type strain, bovine isolate (1)	Iowa	150	22
	TMC 401 and 412, bovine isolates (2)	Wisconsin, England	150	22
	TMC 409, host unknown (1)	France	150	22
	Bovine isolates (12)	Texas	150	10
	Bovine isolates (6)	Hawaii, Iowa, Maryland, New Mexico, Texas, Virginia	150	5
	Human isolates (28)	California	150	6
	Human isolates (5)	Switzerland	150	5
M. bovis BCG	ATCC 19274, 27290, 35734, 35736, 35746 (5)	Quebec, Brazil, Denmark, France	200	2
	TMC 1002, 1009, 1010, 1012, 1019, 1020, 1021, 1022, 1024, 1025, 1028, 1029, 1030, 1103, 1108 (15)	Illinois, Maryland, New York, Ontario, Quebec, Australia, Czechoslovakia, Denmark, France, Japan, Mexico, Russia, Sweden	200	22
	Human isolate (1)	Tennessee	200	7
	Human isolates (2)	California	200	6, 10

[a] Abbreviations: TMC, Trudeau Mycobacterial Culture Collection; ATCC, American Type Culture Collection.
[b] Geographic locations are listed by state or province within the United States and Canada and by country for other locations. Locations for BCG reference strains refer to the location of the laboratory which transmitted them to the TMC or ATCC.

tuberculosis complex strains, including *M. tuberculosis*, *M. africanum*, and *M. microti* strains.

DISCUSSION

BCG history. BCG has a unique history. From 1908 through 1921, a virulent strain of *M. bovis* isolated from bovine milk was passaged every 15 days alternately on potato bile and Sauton media at the Institut Pasteur (22). The strain was determined to be attenuated and then concurrently maintained on three media: potato bile, potato with glycerin beef broth, and potato Sauton media (9). The early BCG substrains (ATCC 35736, TMC 1009, TMC 1019, and TMC 1022) were distributed between 1925 and 1928. In 1932, the potato bile and potato with glycerin beef broth lines were discontinued. Other BCG substrains were subsequently distributed.

BCG has since been used predominately as a vaccine against tuberculosis. It is also an effective cancer immunotherapy (3). Most recently, BCG has been developed as a recombinant vehicle for multivalent vaccines against other diseases (20). The ability to differentiate BCG from other members of the *M. tuberculosis* complex is important for at least two reasons. First, although less virulent than its parent strain, BCG can cause disease in humans (18, 19). Approximately 5% of patients undergoing intravesical BCG immunotherapy for bladder cancer experienced adverse reactions (15, 16). Accurate identification of BCG will give health care providers important epidemiological and treatment information. Second, the ability to identify BCG in areas where BCG is used and other *M. tuberculosis* complex organisms are prevalent is required to establish the rate of BCG complications. This is especially relevant in the era of AIDS (7, 27).

Distinguishing *M. bovis* strains from other members of the *M. tuberculosis* complex. BCG substrains retain certain genetic characteristics of the parent *M. bovis* strain. Both have a deletion of the MTP40 genetic fragment (23), a specific mutation in the *pncA* gene, conferring pyrazinamide resistance (24), and a specific mutation in the *oxyR* gene (25). Some of these genetic characteristics have been exploited to distinguish *M. bovis* (including BCG) from other species of the *M. tuberculosis* complex (17, 25).

Identification of BCG substrains. Phenotypic heterogeneity among BCG substrains was recognized soon after the initial dissemination from the Institut Pasteur (22). Genetic differences were subsequently identified and have been used to identify substrains. The early BCG substrains differ from late substrains because the former secrete the MPB64 antigen and contain two copies of IS*6110* (1, 9). These early substrains may

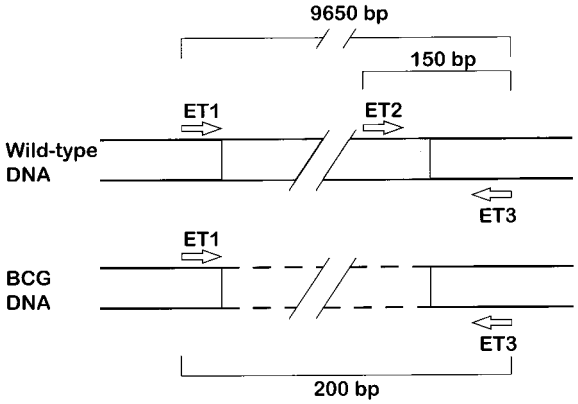

FIG. 1. Multiplex PCR design. Wild-type *M. tuberculosis* complex DNA is represented by the parallel lines in the top diagram. The shaded region contains the 9.5-kb sequence RD1. RD1 is deleted in *M. bovis* BCG DNA, represented by the dashed lines in the lower diagram. The PCR primers ET1, ET2, and ET3 are shown as arrows oriented in the direction of amplification.

have originated from one of the two lines discontinued in 1932. Zhang and his colleagues used large restriction fragment polymorphisms to identify individual substrains (33). This method was used to identify a BCG substrain causing iatrogenic meningitis (26). This method may also be useful for comparisons of the safety and efficacy of the various BCG substrains used for vaccination. However, no criteria have been developed for the identification of the BCG substrains as a group by large restriction fragment polymorphisms.

Significance of the RD1 deletion in BCG substrains. Culture conditions between 1908 and 1921 probably allowed the selection of multiple mutations in the parent *M. bovis* strain, leading to attenuation. Identification and characterization of these mutations are important because all or some may have contributed to the loss of virulence of BCG. Understanding the mechanisms of attenuation may yield insight into virulence mechanisms of *M. tuberculosis*, which may in turn facilitate improvement of our ability to prevent and treat tuberculosis.

Recently, regions of BCG's genetic uniqueness were sought by using subtractive hybridization and DNA sequence comparisons between the BCG and *M. bovis* genomes (21). One region of difference, RD1, encodes a 9.5-kb fragment which contains at least eight open reading frames in *M. bovis*. This region was found to be absent in all BCG strains tested. When the RD1 fragment was cloned into BCG, the resultant recombinant clones expressed many of the encoded proteins, some of which appeared to down-regulate expression of other proteins in this recombinant strain (21).

Although a direct link between this deletion and the attenuation of BCG cannot be established because the virulent parent strain has been lost (9), preliminary data indicate that reintroducing RD1 into BCG may enable BCG to elicit pathology in a manner similar to that of *M. bovis* (21). Therefore, it seems likely that RD1 is associated with the attenuation of BCG. Further characterization of RD1 may facilitate our understanding of BCG attenuation.

In the present study, it was found that RD1 was deleted in all BCG substrains, including recent clinical isolates and early and late reference strains. RD1 was present in all human and bovine *M. bovis* strains from various parts of the world and in other strains of the *M. tuberculosis* complex. These data suggest that this region of the BCG genome is a suitable target for the specific identification of BCG.

Multiplex PCR for rapid detection of BCG strains. The multiplex PCR method for detection of the RD1 deletion was shown to be highly sensitive and specific for the detection of BCG. To evaluate its utility as a diagnostic test, a large collection of *M. tuberculosis* complex strains was analyzed, including 152 isolates from 24 countries. Determining the specificity of the test with multiple *M. bovis* strains was critical, since BCG was derived from *M. bovis* and they are similar genetically. Several of the strains were isolated more than 70 years ago (e.g., TMC 401 and TMC 409 [22]), and many are recent clinical isolates.

Other methods to distinguish BCG from other *M. tuberculosis* complex strains. Several other methods to differentiate BCG from other members of the *M. tuberculosis* complex have been reported. These include phage typing (12); high-performance liquid chromatography (8); restriction fragment length polymorphisms, with the insertion sequence IS*1081* being used as a probe (IS*1081* fingerprinting) (28); amplification of a specific region containing the major polymorphic tandem repeat by PCR, followed by restriction enzyme analysis (MPTR PCR-REA) (10); large restriction fragment polymorphisms (13); and the use of monoclonal antibodies against a 25-kDa antigen (14). The first four of these methods have been well validated with large and diverse strain collections.

The multiplex PCR has significant advantages over the other well-validated methods. Phage typing is laborious and not widely available. High-performance liquid chromatography is rapid and easy to perform but requires a fresh strain as well as specialized and expensive equipment. IS*1081* fingerprinting is laborious, requiring careful DNA isolation from a large bacterial culture followed by Southern hybridization with a specific probe. This is not generally available to clinical microbiology laboratories.

The multiplex PCR method shares some characteristics with the MPTR PCR-REA (10). Both methods have been used successfully on ethanol-fixed mycobacterial cultures. By using the ethanol fixation method, cultures can be rendered noninfectious, be shipped at room temperature, and remain suitable for PCR analysis (31). Both methods involve the use of equipment that is available in most molecular biology laboratories. Both methods base conclusions on the size of a PCR product, not its presence or absence, and so will not give misleading results if inhibitors of PCR are present.

The multiplex PCR method has advantages over the MPTR PCR-REA method (10). The multiplex PCR has been evalu-

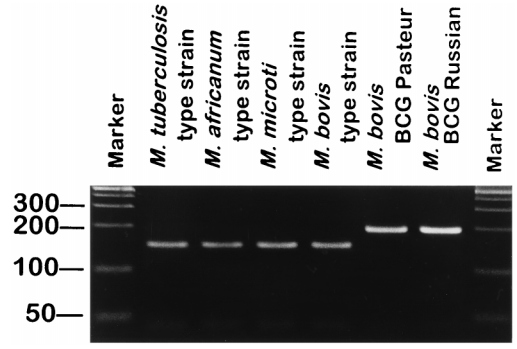

FIG. 2. Ethidium bromide-stained 3% agarose gel of multiplex PCR products. Both early (Russian) and late (Pasteur) *M. bovis* BCG substrains yielded a single 200-bp product, indicating deletion of the RD1 sequence (Fig. 1). A single 150-bp product is apparent for the other four strains shown, indicating the presence of the RD1 sequence. Molecular size markers are shown on the left and right (in base pairs).

ated with a larger collection of relevant strains. The multiplex PCR is completed in a single reaction and analyzed on a single gel, whereas the initial MPTR PCR-REA product must be digested and revisualized on a second gel. The 150- and 200-bp final products of the multiplex PCR are more easily distinguished than the MPTR PCR-REA products. The internal sequences of the 150- and 200-bp products differ and could be distinguished by using a hybridization probe in place of the agarose electrophoresis. Finally, the multiplex PCR method detects a specific, large deletion, whereas the specificity of MPTR PCR-REA depends on a single nucleotide substitution. Theoretically, a nucleotide substitution is more likely to occur spontaneously than a specific, large deletion.

Summary. The multiplex PCR method was 100% sensitive and specific for the identification of BCG among strains of the *M. tuberculosis* complex. RD1 was deleted from all BCG substrains and present in all other strains evaluated. Multiplex PCR can be used as a diagnostic test and has significant advantages over existing methods.

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